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Histone H2A.Z Regulates the Expression of Several Classes of Phosphate Starvation Response Genes But Not as a Transcriptional Activator[1][OA]

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Phosphate (Pi) availability is a major constraint to plant growth. Consequently, plants have evolved complex adaptations to tolerate low Pi conditions. Numerous genes implicated in these adaptations have been identified, but their chromatin-level regulation has not been investigated. The nuclear actin-related protein ARP6 is conserved among all eukaryotes and is an essential component of the SWR1 chromatin remodeling complex, which regulates transcription via deposition of the H2A.Z histone variant into chromatin. Here, we demonstrate that ARP6 is required for proper H2A.Z deposition at a number of Pi starvation response (PSR) genes in Arabidopsis (*Arabidopsis thaliana*). The loss of H2A.Z at these target loci results in their derepression in *arp6* mutants and correlates with the presence of multiple Pi-starvation-related phenotypes, including shortened primary roots and increases in the number and length of root hairs, as well as increased starch accumulation and phosphatase activity in shoots. Our data suggest a model for chromatin-level control of Pi starvation responses in which ARP6-dependent H2A.Z deposition modulates the transcription of a suite of PSR genes.

All living things require phosphorus (P) for survival. Consequently, organisms possess regulatory programs that control the onset of events aimed at facilitating P conservation and/or acquisition during P limitation. The *Saccharomyces cerevisiae PHO* regulon includes a two-component system consisting of regulatory proteins and transcriptional activators that mediates coordinated responses to P limitation (Oshima, 1997). Perception of low P conditions promotes the binding of the basic helix-loop-helix transcription factor, Pho4p, to *PHO* box cis-elements in the promoters of a number of structural genes, including phosphate transporters and phosphatases (Oshima, 1997; Persson et al., 2003; Nishizawa et al., 2008).

For plants, P availability is frequently a major constraint to growth because the concentration of inorganic phosphate (Pi), the plant-available form of P, is extremely low in the soil solution (Marschner, 1995). Therefore, as with yeast, plants have evolved adaptive responses to tolerate low P conditions. Several plant homologs of yeast structural *PHO* genes exist, and the promoters of many plant Pi starvation response (PSR) genes contain cis-elements similar to the yeast *PHO* box (Raghothama, 1999; Hammond et al., 2003). Although no closely related homologs of *PHO* regulatory genes in yeast have been identified in plant genomes, the MYB transcription factors *PHOSPHORUS STARVATION RESPONSE1* and *PHOSPHATE STARVATION RESPONSE1* (*PHR1*) have been identified in *Chlamydomonas reinhardtii* (Wykoff et al., 1999) and Arabidopsis (*Arabidopsis thaliana*; Rubio et al., 2001), respectively, and have been characterized as key activators of many PSR genes. Several recent studies have identified additional transcriptional activators and repressors of Pi responses in plants (Yi et al., 2005; Chen et al., 2007; Devaiah et al., 2007a, 2007b; Duan et al., 2008), and microarray analyses have revealed at least two Pi starvation signaling programs: genes that respond quickly to Pi deficiency and those that respond more slowly to prolonged Pi deficiency (Hammond et al., 2004). Together, these studies point toward an elaborate network employed by plants to cope with Pi starvation.

In eukaryotes, the efficiency of gene transcription is ultimately determined by the higher-order structure of chromatin. Many mechanisms exist to alter chromatin

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structure at promoters and other DNA regulatory elements to modulate both basal and induced transcription rates. A number of studies have shown that several chromatin-level mechanisms are involved in regulating the transcription of some yeast structural *PHO* genes. For example, the SWI/SNF nucleosome remodeling complex and the Gcn5 histone acetyltransferase are necessary for full induction of several phosphatase genes (*PHO5*, *PHO8*, *PHO11*, and *PHO12*) and the *PHO84* high-affinity phosphate transporter gene (Santisteban et al., 2000; Sudarsanam et al., 2000; Barbaric et al., 2007; Wippo et al., 2009). The SWR1 complex, which regulates transcription through the deposition of the H2A.Z histone variant at target genes, has also been implicated in modulating the transcription of a number of *PHO* genes (Santisteban et al., 2000; Lindstrom et al., 2006).

In contrast to yeast, the involvement of chromatin-level mechanisms in the regulation of plant PSR genes has not been investigated. Recently, several studies have indicated that the SWR1 complex described in other eukaryotes is conserved in Arabidopsis (March-Diaz and Reyes, 2009). In addition, the Arabidopsis nuclear actin-related protein ARP6 has been identified as a key component of SWR1 and is required for normal deposition of histone H2A.Z at target loci (Choi et al., 2007; Deal et al., 2007). As a result, plants defective in ARP6 exhibit diverse developmental phenotypes. For example, mutation of *ARP6* results in altered leaf, inflorescence, and flower development and early flowering (Choi et al., 2005; Deal et al., 2005). The early flowering phenotype is due, in part, to disruption of ARP6-dependent H2A.Z deposition at the floral repressor genes *FLOWERING LOCUS C*, *MADS AFFECTING FLOWERING4* (*MAF4*), and *MAF5* (Deal et al., 2007). Previously, we observed that *arp6* mutant seedlings also exhibit altered root growth similar to that of wild-type Arabidopsis grown under Pi-limiting conditions (A.P. Smith and A. Jain, unpublished data). Thus, ARP6 may be involved in modulating PSRs by regulating expression of key PSR genes.

The goal of this study was to investigate the role of ARP6-dependent H2A.Z deposition in controlling PSRs in Arabidopsis. We report that mutation of *ARP6* results in a dramatic decrease in H2A.Z abundance at a number of PSR genes from diverse functional categories. The loss of H2A.Z is accompanied by increases in gene transcription (i.e. derepression) of the PSR genes and the manifestation of multiple PSRs in *arp6* mutant seedlings. Our data support a role for the SWR1 complex in repressing the onset of PSRs by modulating the expression of a number of PSR genes.

RESULTS

arp6 Mutants Exhibit PSRs under Pi-Replete Conditions

Plants have evolved complex adaptations to tolerate low Pi conditions. Some of the most prominent PSRs are changes in root morphology aimed at mining additional Pi from soil (Franco-Zorrilla et al., 2004). These include increases in the number and length of root hairs and a decrease in primary root growth (Ma et al., 2001; Sanchez-Calderon et al., 2005; Jain et al., 2007). In preliminary experiments, we observed that *arp6* mutant seedlings exhibited altered root growth similar to that of Pi-starved wild type. To initiate an investigation of the potential role of ARP6 in modulating PSRs, seedlings of the *arp6* T-DNA mutants *arp6-1* and *arp6-2* (Deal et al., 2005) were grown under Pi-replete conditions and were screened for traits indicative of Pi starvation at 7 or 12 d old. Seedlings of both *arp6-1* and *arp6-2* had approximately 2.5-fold more root hairs relative to the wild type in a 5-mm section from the primary root apex, and these hairs were 2-fold longer on average (Fig. 1, A and B). In addition, both *arp6* mutants exhibited reduced primary root growth relative to the wild type (Fig. 1C). Other PSRs include increased starch accumulation, which results from low Pi-induced alteration of primary metabolism (Amtmann et al., 2006), and increased acid phosphatase activity, which aims to remobilize intracellular Pi (del Pozo et al., 1999; Li et al., 2002). In shoots of the *arp6* mutants, we observed increases in both starch accumulation (Fig. 1D) and acid phosphatase activity (Fig. 1E) compared to the wild type. Together, these data suggest the onset of PSRs in *arp6* mutant seedlings despite being grown under Pi-replete conditions. To determine if the PSRs in the mutants correlate with a defect in cellular Pi status, we compared the soluble Pi contents of *arp6-1*, *arp6-2*, and the wild type. In both shoots and roots, *arp6-1* and *arp6-2* seedlings contained moderately less Pi compared to the wild type (Fig. 2).

Molecular Phenotypes of *arp6* Mutant Seedlings

To gain insight into the factors influencing the Pi-starvation phenotypes we observed in *arp6* mutants, we used quantitative reverse transcription-PCR to compare the transcript levels from a number of PSR genes (Misson et al., 2005; Bari et al., 2006) among 12-d-old wild-type, *arp6-1*, and *arp6-2* seedlings. Specifically, we examined six different functional categories of Pi-starvation-induced genes, including those involved in Pi transport, Pi distribution, signal transduction, primary metabolism (e.g. lipid and starch metabolism), and protein turnover (Table I). First, we quantified the Pi starvation induction of these genes in both shoots and roots of wild-type seedlings. Transcript levels for all 10 genes were significantly higher (i.e. derepressed) in both tissues of Pi-starved wild type (i.e. 5 d on normal media followed by 7 d on Pi-deficient media; see "Materials and Methods") compared to the wild type grown under Pi-replete conditions (Table I), corroborating data from previous studies (Misson et al., 2005; Bari et al., 2006). Next, we quantified the expression of these genes in the wild type, *arp6-1*, and *arp6-2* grown under Pi-replete conditions. Transcript levels for all but two of the 10 genes were at least 2-fold greater in

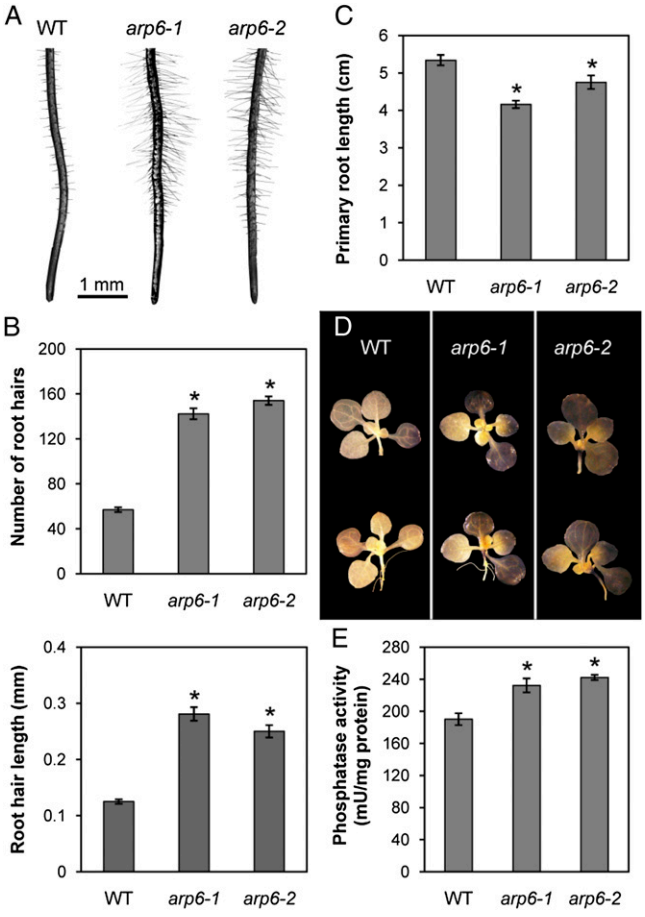

Figure 1. *arp6* mutants exhibit Pi starvation symptoms under Pi-replete conditions. Data for 7- (A and B) and 12-d-old (C–E) wild-type (WT), *arp6-1*, and *arp6-2* seedlings are shown. A and B, Development of root hairs in a 5-mm section from the primary root tip. Bar = 1 mm. C, Primary root length. B and C, Values are the mean ± SE; $n = 20$ (10 seedlings from each of two independent experiments). D, Representative iodine-stained shoots of 10 seedlings each of the wild type and the *arp6* mutants showing starch accumulation. E, Phosphatase activity in the shoots of the wild type and the *arp6* mutants. Values are the mean ± SE; $n = 9$ replicates of 150 to 200 seedlings each. Bars with asterisks are significantly different from the wild type (*t* test, $P \leq 0.05$).

arp6-1 and *arp6-2* than in the wild type in at least one tissue (i.e. shoot or root; Fig. 3). For instance, expression of *At4*, which is involved in Pi distribution (Shin et al., 2006), was substantially greater (from 21- to 83-fold) in shoots and roots of both *arp6* mutants compared to the wild type. In addition, the shoot expression of β-amylase (*BMY1*), which plays a role in starch metabolism (Mita et al., 1997; Niewiadomski et al., 2005) was approximately 5-fold higher in both mutants relative to the wild type. This correlates with the increased starch accumulation observed in *arp6-1* and *arp6-2* shoots grown under Pi-replete conditions (Fig. 1D). Taken together, these results suggest that ARP6 plays a significant role in regulating PSRs in Arabidopsis.

Pi signaling pathways in plants are largely unknown, although some regulatory factors have been identified. A subcomponent of the Arabidopsis Pi signaling network involves the key MYB factor PHR1 as a transcriptional activator of a microRNA, *miR399*, which regulates the E2 conjugase, PHO2. PHO2, in turn, controls expression of a subset of PSR genes, including *At4*, *IPS1*, *Pht1;8*, and *Pht1;9* (Bari et al., 2006). One explanation for the derepression of multiple PSR genes in *arp6* mutants could be a defect in the PHR1-miR399-PHO2 signaling pathway. To test this, we compared the expression of *PHR1*, *miR399* (via analysis of one of the five miR399 primary transcripts, *miR399c*; Bari et al., 2006), and *PHO2* among the wild type, *arp6-1*, and *arp6-2*. As shown in Figure 4, we saw no significant changes (<2-fold) in transcript levels for these genes in either shoots or roots between the wild type and the *arp6* mutants. This suggests that the PHR1-miR399-PHO2 signaling pathway does not play a major role in the derepression of PSR genes in *arp6-1* and *arp6-2* under Pi-replete conditions.

PSR Genes Are Targets of H2A.Z Histone Variant Deposition

Arabidopsis ARP6 participates in a SWR1-like chromatin remodeling complex that deposits the H2A.Z histone variant into chromatin (Deal et al., 2007;

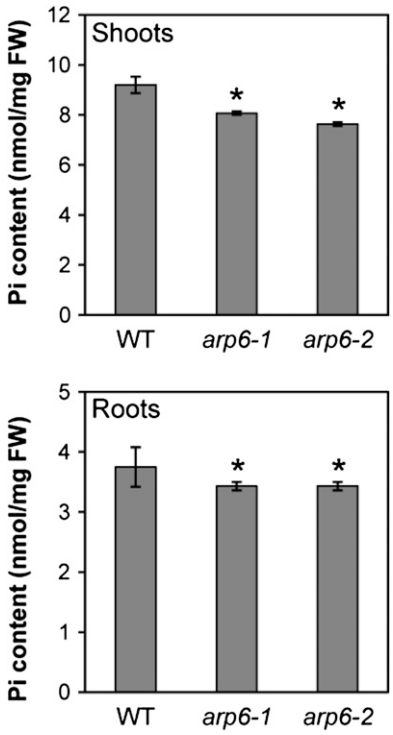

Figure 2. *arp6* mutants accumulate less Pi compared to the wild type. Soluble Pi levels are shown for shoots and roots of 12-d-old wild-type (WT), *arp6-1*, and *arp6-2* seedlings grown under Pi-replete conditions. Values are the mean ± SE; $n = 9$ replicates of 150 to 200 seedlings each. Bars with asterisks are significantly different from the wild type (*t* test, $P \leq 0.05$). FW, Fresh weight.

Table I. *Pi starvation induction of PSR genes in wild-type seedlings*

Relative quantity (RQ) values are the mean ± SE from three technical replicates where the fold inductions were calculated by normalizing expression from Pi-starved tissues (P−) to expression from the wild type grown under Pi-replete conditions (P+).

Functional Category	Transcript	Annotation	Fold Induction (P−/P+)					
			Shoot			Root		
			RQ	SE		RQ	SE	
				Mininum	Maximum		Mininum	Maximum
Pi transport	At5g43350	Pht1;1	2.8	0.6	0.7	2.5	0.1	0.1
	At5g43370	Pht1;2	239.6	46.0	56.9	454.8	17.3	18.0
	At2g38940	Pht1;4	7.4	1.4	1.7	5.0	0.2	0.2
	At1g76430	Pht1;9	222.0	41.7	51.4	26.9	1.1	1.2
Pi distribution	At5g03545	At4	93.3	5.3	5.6	33.0	5.0	5.9
Signal transduction	At5g20150	SPX1	44.3	2.0	2.1	20.3	4.7	6.1
Primary metabolism	At4g15210	β-amylase (BMY1)	16.0	3.1	3.8	243.9	46.9	58.0
	At3g02040	Glycerophosphodiester phosphodiesterase (SRG3)	25.9	1.1	1.1	14.3	2.9	3.6
	At2g36970	UDP-glucuronosyl/UDP-glucosyl transferase	12.3	0.5	0.6	10.1	2.1	2.7
Protein turnover	At4g34210	Arabidopsis SKP1-like 11 (ASK11)	8.0	1.5	1.9	4.4	1.3	1.8

March-Diaz et al., 2007). To determine whether PSR genes are directly targeted by ARP6-dependent H2A.Z deposition, we performed chromatin immunoprecipitation (ChIP) analyses on 10 PSR genes in 12-d-old wild-type and *arp6-1* seedlings using Arabidopsis H2A.Z antibodies. ChIP with H2A.Z antibodies significantly enriched all 10 loci in both shoots and roots from the wild type compared to control antisera but did not enrich these loci from *arp6-1* tissues (Fig. 5). These results indicate that H2A.Z is deposited at all the PSR genes tested under normal conditions and that ARP6 is required for this deposition.

If H2A.Z is involved in the regulation of PSR genes, its presence at target loci may be dependent on the Pi status of the plant. To test this, we used ChIP analysis to compare the abundance of H2A.Z at the 10 PSR genes between Pi-replete and Pi-starved wild-type seedlings. Again, H2A.Z antibodies significantly enriched all 10 loci in Pi-replete wild type; however, the H2A.Z enrichment for five of the genes was significantly lower in Pi-starved wild type (Fig. 6). This indicates that the levels of H2A.Z at a subset of the PSR genes examined were lower during transcriptional activation than under repressive conditions.

DISCUSSION

As in other eukaryotes, the SWR1 chromatin remodeling complex is required for deposition of histone H2A.Z at target loci in Arabidopsis (Choi et al., 2007; Deal et al., 2007; March-Diaz and Reyes, 2009), and examination of H2A.Z localization genome-wide suggests that this histone variant is involved in the modulation of numerous genes (Zilberman et al., 2008). The nuclear actin-related protein ARP6 has been identified as a key component of SWR1, and as a result, mutation of *ARP6* results in a pleiotropic phenotype, causing a variety of defects, including altered development of leaf, inflorescence, and flower, as well as early flowering (Choi et al., 2005; Deal et al., 2005). In contrast, ARP6 overexpression lines are phenotypically identical to the wild type with respect to flowering time, leaf, root, inflorescence, and flower development, and fertility (Deal et al., 2005; R.B. Deal, A.P. Smith, and R.B. Meagher, unpublished data). This is likely due to the fact that ARP6 activity is limited by other components of the SWR1 complex. A similar phenomenon has been observed for another SWR1-complex factor, SERRATED AND EARLY FLOWERING/AtSWC6. Loss of *AtSWC6* causes many of the same developmental defects as mutation of *ARP6* (Choi et al., 2007; March-Diaz et al., 2007), yet *AtSWC6* overexpression also confers no visible or molecular phenotypes (Choi et al., 2007).

The early flowering phenotype in *arp6* mutants is due, in part, to disruption of ARP6-dependent H2A.Z deposition at several floral repressor genes (Deal et al., 2007). Preliminary microarray experiments comparing wild-type and *arp6-1* plants reveal large numbers of genes that are either up-regulated, down-regulated, or unchanged (data not shown). However, further examination of a subset of genes whose expression was reduced in *arp6-1*, including a *CONSTANS-LIKE* transcription factor, the *WRK70* transcription factor, and the putative disease resistance genes *PRB1* and *TIR*, indicated that even in wild-type plants these loci did not accumulate significant amounts of H2A.Z (Deal et al., 2007), suggesting that these and perhaps many other genes are not directly regulated by ARP6-dependent H2A.Z deposition. Here, we demonstrate that mutation of *ARP6* results in many morphophysiological and molecular phenotypes indicative of PSRs. Together, these phenotypes, along with our expression and ChIP analyses, suggest that ARP6 directly modulates the manifestation of PSRs through its role in the SWR1 complex, which deposits H2A.Z at a number of PSR genes.

Data from a wealth of studies support a positive role for H2A.Z in transcription by poising genes for acti-

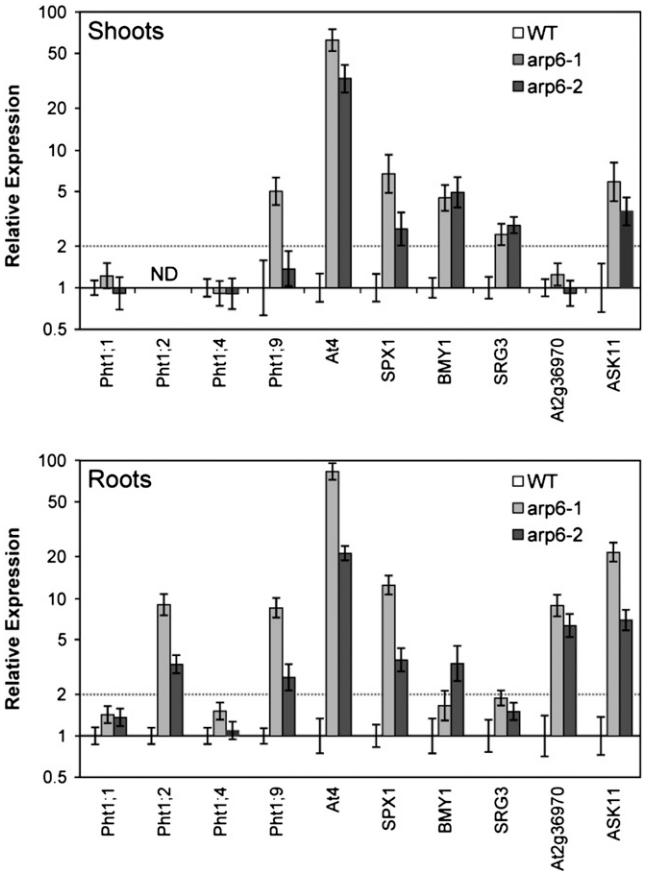

Figure 3. Multiple PSR genes are misregulated in *arp6* mutants. The tissues (shoots and roots) examined were harvested from 12-d-old seedlings of the wild type (WT), *arp6-1*, and *arp6-2* grown under Pi-replete conditions. Values are plotted on a log scale and are the mean ± SE from two biological replicates (of three technical replicates each) where the fold changes were normalized to transcript levels in the wild type. ND, no data; *Pht1;2* transcripts in shoot tissues were below the limit of detection in these experiments.

vation (Guillemette and Gaudreau, 2006). In contrast, here, we show that loss of H2A.Z at many PSR genes coincides with their derepression, suggesting a negative role for H2A.Z in gene regulation. This phenomenon may result from an overriding transcriptional activation mechanism(s) initiated by Pi starvation of *arp6* mutant tissues that does not require H2A.Z. However, the Pi levels in *arp6* tissues were 83 to 91% of wild-type levels (Fig. 2), consistent with the *arp6* mutants experiencing only a small Pi starvation stress. Alternatively, H2A.Z may play a repressive role in the transcription of some PSR genes. Gene expression studies in H2A.Z-deficient mutants of *S. cerevisiae* (*htz1Δ*; Meneghini et al., 2003) and Arabidopsis (*hta9/hta11*; March-Diaz et al., 2008) identified substantial numbers of genes that were either up-regulated or down-regulated, suggesting that H2A.Z plays both repressive and inductive roles in transcription. In addition, our ChIP data show that for a subset of PSR genes examined, the levels of H2A.Z were higher at loci in Pi-replete tissues compared to Pi-starved tissues (Fig. 6). Other studies have also shown that H2A.Z is more abundant at some target loci under repressive conditions than during activation (Guillemette et al., 2005; Raisner et al., 2005; Deal et al., 2007). Furthermore, H2A.Z has been implicated in the repression of a number of stress response genes in yeast (Lindstrom et al., 2006) and in the repression of the systemic acquired resistance response in Arabidopsis (March-Diaz et al., 2008). Therefore, under normal conditions (i.e. Pi replete), ARP6-mediated H2A.Z deposition may act to maintain some PSR genes in a repressed transcriptional state.

Although H2A.Z appears to have a negative impact on PSR gene expression, the associated mechanisms are unclear. Under normal conditions, H2A.Z-containing nucleosomes might create a local chromatin structure at target loci that is not conducive to gene expression, perhaps by interfering with the binding of transcriptional activators and/or by promoting transcriptional repressor binding. A number of both positive and negative PSR-related transcription factors are known (Rubio et al., 2001; Yi et al., 2005; Chen et al., 2007; Devaiah et al., 2007a, 2007b; Duan et al., 2008), and their activities could be dependent on local chromatin environments influenced by H2A.Z. Our molecular analyses demonstrated that transcript levels for *PHR1*, *miR399*, and *PHO2* were unchanged in *arp6* mutants. However, *PHR1* expression is not highly responsive to Pi starvation even though it plays a key role in activating downstream PSR genes (Rubio et al., 2001). Therefore, our analyses do not rule out the possibility that loss of H2A.Z in *arp6* mutants impacts the interaction of the PHR1 protein with the promoters of H2A.Z-targeted PSR genes. Nucleosome remodeling at the promoters of several yeast *PHO*

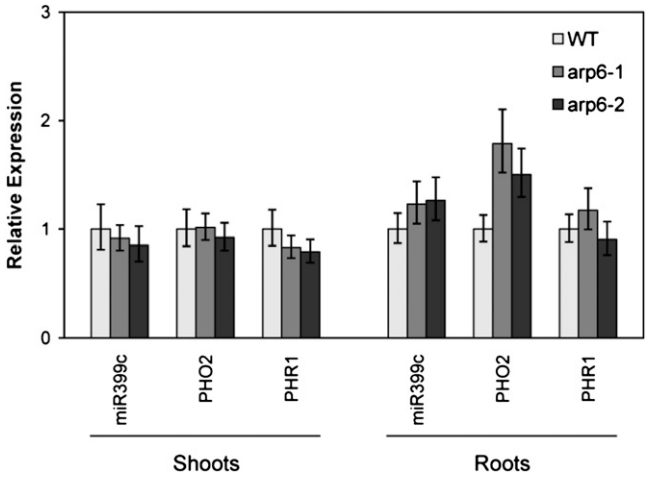

Figure 4. Expression of PHR1-miR399-PHO2 signaling pathway components in *arp6* mutants. The tissues (shoots and roots) examined were harvested from 12-d-old seedlings of the wild type (WT), *arp6-1*, and *arp6-2* grown under Pi-replete conditions. Values are the mean ± SE from two biological replicates (of three technical replicates each) where the fold changes were normalized to transcript levels in the wild type.

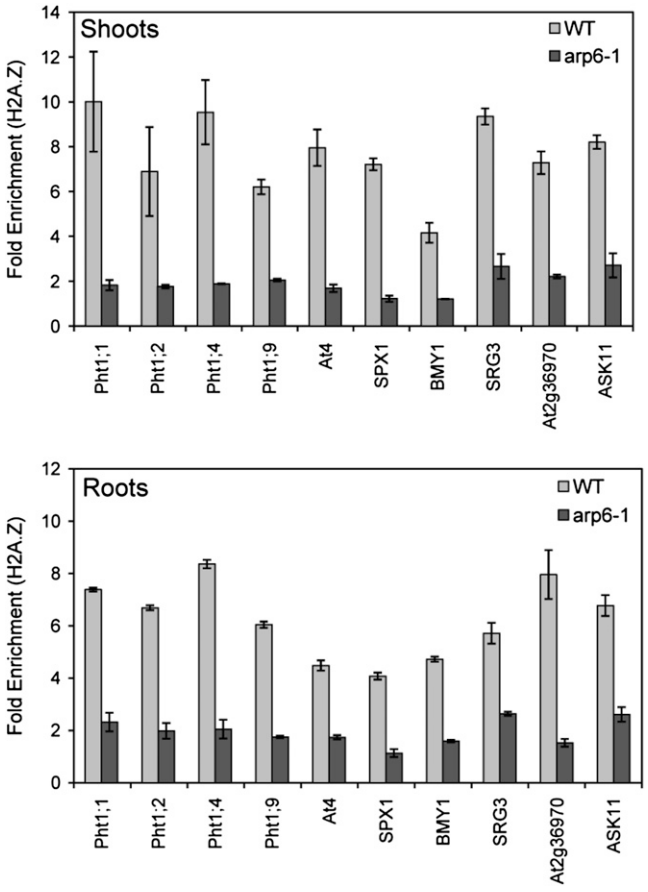

Figure 5. ARP6 is required for H2A.Z deposition at a suite of PSR genes. The abundance of histone variant H2A.Z was assayed in12-d-old seedlings (shoots and roots) of the wild type (WT) and *arp6-1* for 10 PSR genes using a ChIP assay with H2A.Z antibodies. The values are the average fold enrichment of DNA ± SD as measured by real-time PCR of six replicates from two independent experiments.

genes (e.g. *PHO5*, *PHO8*, and *PHO84*) occurs in response to Pi starvation and exposes cis-elements recognized by the Pho4p transcriptional activator (Wippo et al., 2009). A similar mechanism that involves H2A.Z may be in place for the regulation of Arabidopsis PSR genes. However, a direct comparison cannot be made since the Arabidopsis PSR genes examined in this study are derepressed in H2A.Z-defective plants (i.e. *arp6* mutants), whereas loss of H2A.Z results in the down-regulation of the yeast *PHO* genes (Santisteban et al., 2000; Lindstrom et al., 2006). Future nucleosome occupancy (i.e. nuclease protection) assays may reveal the relationship between nucleosome positioning and exposure of putative Pi-responsive cis-elements in the promoters of Arabidopsis PSR genes. Recently, SPX1 was shown to positively regulate expression of a subset of PSR genes (Duan et al., 2008). Our data show that *SPX1* is a direct target of H2A.Z deposition and its expression is elevated in *arp6* mutants. Therefore, ARP6-dependent H2A.Z deposition likely affects modulation of PSRs both by regulating structural Pi-starvation-induced genes and by controlling Pi signaling components that impact the expression of multiple targets.

The SWR1 complex typically has overlapping and/or cooperative functions with various chromatin cofactors, including other chromatin remodeling complexes and histone modifying enzymes (Santisteban et al., 2000; Li et al., 2005; Lindstrom et al., 2006). The expression of yeast *PHO5*, *PHO8*, and *PHO84* all have requirements for the SWI/SNF remodeling complex, the Gcn5 histone acetytltransferase, and the Asf1 histone chaperone, but to considerably different degrees (Barbaric et al., 2007; Wippo et al., 2009). Microarray analyses have also implicated SWR1, the NuA4 histone acetyltransferase complex, and the Isw1 chromatin-remodeling enzyme in the regulation of these and other *PHO* genes (Lindstrom et al., 2006). In addition, regulation of gene transcription by H2A.Z is influenced by the ability of H2A.Z to antagonize DNA methylation-induced gene silencing (Zilberman et al., 2008). The fact that H2A.Z is one of many components that regulates gene transcription likely explains the observation that although H2A.Z is found genome-wide, its loss affects the transcription of only a subset of the genome (Lindstrom et al., 2006; Zilberman et al., 2008). This notion is supported by our data showing that the expression of some H2A.Z-targeted PSR genes (e.g. *Pht1;1* and *Pht1;4*) is unchanged in *arp6* mutants (compare Figs. 3 and 5). Differences in the relative PSR gene transcript levels in *arp6* tissues (Fig. 3) compared to their levels in Pi-starved wild type (Table I) further suggest varying dependence of PSR gene promoters on H2A.Z. This may also reflect differences in the extent to which H2A.Z modulates basal and induced expression at distinct genes. Our ChIP data comparing

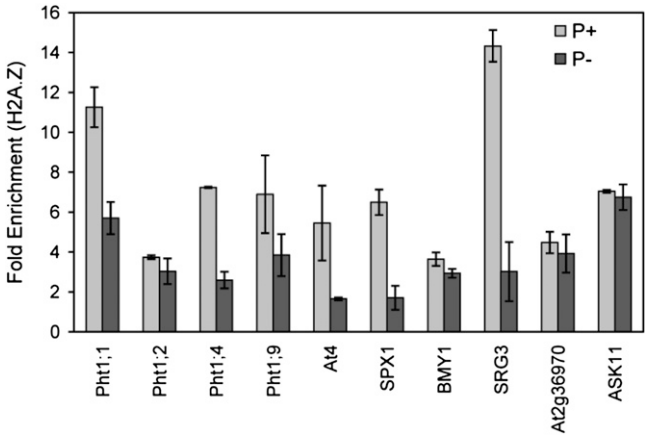

Figure 6. The presence of histone variant H2A.Z at many PSR genes differs between Pi-replete and Pi-starved wild type. The abundance of histone variant H2A.Z was assayed at 10 PSR genes using a ChIP assay with H2A.Z antibodies. The tissues examined were harvested from wild-type seedlings grown hydroponically for 12 d in complete nutrient media and transferred either to fresh nutrient media (P+) or to nutrient media lacking Pi (P−) for an additional 2 d. The values are the average fold enrichment of DNA ± SD as measured by real-time PCR of six replicates from two independent experiments.

Pi-replete and Pi-starved wild type, which show a difference in H2A.Z abundance at only some of the PSR genes tested, provide additional evidence for promoter specificity in regard to regulation by H2A.Z (Fig. 6). Alternatively, the Pi starvation treatment employed in our study may not yield maximal induction for all PSR genes. Future investigation of PSR gene transcription in *arp6* mutants grown under different Pi concentrations, as well as examination of genetic interactions between SWR1 components and other chromatin cofactors, will help to elucidate the relative importance of H2A.Z in controlling basal and Pi-starvation-induced PSR gene transcription.

Our data indicate that ARP6 is required for proper H2A.Z deposition at a number of diverse PSR genes, suggesting that H2A.Z plays a significant role in modulating responses to Pi starvation in Arabidopsis. This phenomenon shares similarities with the *PHO* regulatory system in yeast, where transcription of many structural genes is regulated by H2A.Z. An important distinction is that loss of H2A.Z results in the down-regulation of yeast *PHO* genes but a derepression of the Arabidopsis PSR genes. However, a microarray study in yeast revealed that at least one *PHO* gene encoding a phosphate transporter (Pho87) is derepressed in the *H2A.Z* (*htz1*) mutant (Lindstrom et al., 2006). In addition, loss of Set1, the catalytic subunit of a histone methylation complex, results in the derepression of *PHO5* and *PHO84* (Carvin and Kladde, 2004). These studies suggest that gene repression by chromatin cofactors plays a role in modulating PSRs in Arabidopsis, as well as in yeast. It is possible that both yeast and plants utilize chromatin-level mechanisms, such as SWR1, to repress multiple stress-induced pathways under normal conditions. Mutation of yeast *SWR1* and/or *H2A.Z* (*HTZ1*) results in the derepression of a large number of stress-induced genes, including many heat shock genes (Lindstrom et al., 2006). In addition, mutation of *PIE1*, the Arabidopsis *SWR1* homolog, results in the derepression of numerous stress-responsive genes (March-Diaz et al., 2008). A number of these genes are defense related, implicating the SWR1 complex in the repression of the systemic acquired resistance response (March-Diaz et al., 2008). Interestingly, of 621 mis-regulated genes in the *pie1* mutant, 67 were also found to be misregulated in response to Pi starvation (Misson et al., 2005). This further supports our data and points toward a role for SWR1-dependent H2A.Z deposition in the modulation of diverse PSRs in Arabidopsis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0, as well as *arp6-1* and *arp6-2* in the Columbia-0 background (Deal et al., 2005), were used in this study. Seeds were surface sterilized and grown under a 16/8-h photoperiod at 22°C with an average photosynthetically active radiation of 60 μmol m^{-2} s^{-1} provided by fluorescent tubes. For the ChIP experiment comparing Pi-replete and Pi-starved wild type, seedlings were grown hydroponically in a modified Murashige and Skoog (MS) medium containing 1.25 mM KH_2PO_4, pH 5.7, and 1.5% (w/v) Suc for 12 d and were transferred to fresh medium or Pi-deficient medium, which was the modified MS medium containing K_2SO_4 substituted for KH_2PO_4, for an additional 2 d. For all other experiments, seedlings were grown vertically in plates containing solid medium. Seeds were first germinated on plates containing half-strength MS medium, 1.2% (w/v) agar (A-1296; Sigma-Aldrich), and 1.5% (w/v) Suc for 5 d. Uniformly grown seedlings (with primary root length in the range of 1.5–2.5 cm) were transferred to the modified MS medium containing 1.25 mM KH_2PO_4, pH 5.7, and 1.5% (w/v) Suc and were grown for an additional 2 or 7 d, as stated. These are referred to as 7- and 12-d-old seedlings. For Pi-deficient medium, seedlings were transferred to a modified MS medium containing K_2SO_4 substituted for KH_2PO_4.

Seedling Morphological Analysis

For root hair analysis, images of the root hairs growing in the 5-mm section from the tip of the primary root of 7-d-old seedlings were captured using a stereomicroscope (Nikon SMZ-U). For documentation of primary root length, 12-d-old seedlings were scanned in a transmissive mode directly from the petri plates using a desktop scanner (UMAX Powerlook 2100 XL), and ImageJ (a Java image-processing program; http://rsb.info.nih.gov/ij) was used for measurement.

Soluble Pi Content

Shoot and roots were separated, rinsed in distilled water, blotted dry, frozen, and ground to a fine powder in liquid nitrogen. Approximately 20 to 25 mg of the ground tissue was suspended in 250 mL of 1% glacial acetic acid, vortexed, and centrifuged to pellet the cellular debris. The supernatant was assayed for Pi using a phosphomolybdate colorimetric assay (Ames, 1966).

Acid Phosphatase Activity

Shoots from 12-d-old seedlings were excised and ground to a powder in liquid nitrogen. Between 40 and 50 mg of frozen powder was used for the assays. Acid phosphatase activity was quantified by a *p*-nitrophenyl phosphate hydrolysis assay based on spectrophotometric measurement at 405 nm (Richardson et al., 2001) and is expressed as mU/mg protein. Total protein was estimated separately using Bradford's reagent.

Starch Accumulation Analysis

Leaves from 12-d-old seedlings were excised, soaked in 95% ethanol overnight, and rinsed twice with water. Accumulation of starch was visualized by staining with iodine solution. The iodine solution was made by combining 500 μL of 300 mM KI (final concentration 6 mM) and 500 μL of 0.1 N iodine (Aldrich iodine volumetric standard) to a final volume of 10 mL. The tissues were stained for 5 min and rinsed with water. Images were captured under the stereomicroscope.

Quantitative Reverse Transcription-PCR

Twelve-day-old seedlings were separated into roots and shoots. Total RNA was isolated using the Qiagen RNeasy plant mini kit. RNA was treated with RQ1 RNase-free DNase I (Promega) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed with the SuperScript III first-strand synthesis kit (Invitrogen). Real-time PCR was performed with an Applied Biosystems 7500 real-time PCR system using SYBR Green detection chemistry (Applied Biosystems) and gene-specific primers. The 40S ribosomal protein S16 gene (*RPS16A*; *At2g09990*) was used as an internal control, and the relative expression levels of the genes were computed by the 2^{-ddCt} method of relative quantification (Livak and Schmittgen, 2001). Oligonucleotide primer sequences are available upon request.

ChIP

ChIP was performed as described (Gendrel et al., 2005). For each experiment, 1 to 2 g of tissue (12-d-old shoots or roots or 14-d-old hydroponically grown whole seedlings) was used. The affinity-purified H2A.Z antibody was

used at a dilution of 1:100 (Deal et al., 2007). DNA was analyzed by quantitative real-time PCR with the *ACT2* (*At3g18780*) 3′ untranslated region sequence as the endogenous control. The relative quantity value, calculated by the 2^{-ddCt} method (Livak and Schmittgen, 2001), was reported as fold enrichment.

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LITERATURE CITED

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol **8:** 115–118

Amtmann A, Hammond JP, Armengaud P, White PJ (2006) Nutrient sensing and signalling in plants: potassium and phosphorus. Adv Bot Res **43:** 209–257

Barbaric S, Luckenbach T, Schmid A, Blaschke D, Horz W, Korber P (2007) Redundancy of chromatin remodeling pathways for the induction of the yeast PHO5 promoter in vivo. J Biol Chem **282:** 27610–27621

Bari R, Pant BD, Stitt M, Scheible WR (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol **141:** 988–999

Carvin CD, Kladde MP (2004) Effectors of lysine 4 methylation of histone H3 in *Saccharomyces cerevisiae* are negative regulators of PHO5 and GAL1-10. J Biol Chem **279:** 33057–33062

Chen ZH, Nimmo GA, Jenkins GI, Nimmo HG (2007) BHLH32 modulates several biochemical and morphological processes that respond to Pi starvation in Arabidopsis. Biochem J **405:** 191–198

Choi K, Kim S, Kim SY, Kim M, Hyun Y, Lee H, Choe S, Kim SG, Michaels S, Lee I (2005) SUPPRESSOR OF FRIGIDA3 encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in *Arabidopsis*. Plant Cell **17:** 2647–2660

Choi K, Park C, Lee J, Oh M, Noh B, Lee I (2007) Arabidopsis homologs of components of the SWR1 complex regulate flowering and plant development. Development **134:** 1931–1941

Deal RB, Kandasamy MK, McKinney EC, Meagher RB (2005) The nuclear actin-related protein ARP6 is a pleiotropic developmental regulator required for the maintenance of FLOWERING LOCUS C expression and repression of flowering in *Arabidopsis*. Plant Cell **17:** 2633–2646

Deal RB, Topp CN, McKinney EC, Meagher RB (2007) Repression of flowering in *Arabidopsis* requires activation of FLOWERING LOCUS C expression by the histone variant H2A.Z. Plant Cell **19:** 74–83

del Pozo JC, Allona I, Rubio V, Leyva A, de la Pena A, Aragoncillo C, Paz-Ares J (1999) A type 5 acid phosphatase gene from *Arabidopsis thaliana* is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. Plant J **19:** 579–589

Devaiah BN, Karthikeyan AS, Raghothama KG (2007a) WRKY75 transcription factor is a modulator of phosphate acquisition and root development in Arabidopsis. Plant Physiol **143:** 1789–1801

Devaiah BN, Nagarajan VK, Raghothama KG (2007b) Phosphate homeostasis and root development in Arabidopsis are synchronized by the zinc finger transcription factor ZAT6. Plant Physiol **145:** 147–159

Duan K, Yi K, Dang L, Huang H, Wu W, Wu P (2008) Characterization of a subfamily of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. Plant J **54:** 965–975

Franco-Zorrilla JM, Gonzalez E, Bustos R, Linhares F, Leyva A, Paz-Ares J (2004) The transcriptional control of plant responses to phosphate limitation. J Exp Bot **55:** 285–293

Gendrel AV, Lippman Z, Martienssen R, Colot V (2005) Profiling histone modification patterns in plants using genomic tiling microarrays. Nat Methods **2:** 213–218

Guillemette B, Bataille AR, Gevry N, Adam M, Blanchette M, Robert F, Gaudreau L (2005) Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning. PLoS Biol **3:** e384

Guillemette B, Gaudreau L (2006) Reuniting the contrasting functions of H2A.Z. Biochem Cell Biol **84:** 528–535

Hammond JP, Bennett MJ, Bowen HC, Broadley MR, Eastwood DC, May ST, Rahn C, Swarup R, Woolaway KE, White PJ (2003) Changes in gene expression in Arabidopsis shoots during phosphate starvation and the potential for developing smart plants. Plant Physiol **132:** 578–596

Hammond JP, Broadley MR, White PJ (2004) Genetic responses to phosphorus deficiency. Ann Bot (Lond) **94:** 323–332

Jain A, Poling MD, Karthikeyan AS, Blakeslee JJ, Peer WA, Titapiwatanakun B, Murphy AS, Raghothama KG (2007) Differential effects of sucrose and auxin on localized phosphate deficiency-induced modulation of different traits of root system architecture in Arabidopsis. Plant Physiol **144:** 232–247

Li B, Pattenden SG, Lee D, Gutierrez J, Chen J, Seidel C, Gerton J, Workman JL (2005) Preferential occupancy of histone variant H2AZ at inactive promoters influences local histone modifications and chromatin remodeling. Proc Natl Acad Sci USA **102:** 18385–18390

Li DP, Zhu HF, Liu KF, Liu X, Leggewie G, Udvardi M, Wang DW (2002) Purple acid phosphatases of *Arabidopsis thaliana* - Comparative analysis and differential regulation by phosphate deprivation. J Biol Chem **277:** 27772–27781

Lindstrom KC, Vary JC Jr, Parthun MR, Delrow J, Tsukiyama T (2006) Isw1 functions in parallel with the NuA4 and Swr1 complexes in stress-induced gene repression. Mol Cell Biol **26:** 6117–6129

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods **25:** 402–408

Ma Z, Bielenberg DG, Brown KM, Lynch JP (2001) Regulation of root hair density by phosphorus availability in *Arabidopsis thaliana*. Plant Cell Environ **24:** 459–467

March-Diaz R, Garcia-Dominguez M, Florencio FJ, Reyes JC (2007) SEF, a new protein required for flowering repression in Arabidopsis, interacts with PIE1 and ARP6. Plant Physiol **143:** 893–901

March-Diaz R, Garcia-Dominguez M, Lozano-Juste J, Leon J, Florencio FJ, Reyes JC (2008) Histone H2A.Z and homologues of components of the SWR1 complex are required to control immunity in Arabidopsis. Plant J **53:** 475–487

March-Diaz R, Reyes JC (2009) The beauty of being a variant: H2A.Z and the SWR1 complex in plants. Mol Plant **2:** 565–577

Marschner H (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic Press, Boston

Meneghini MD, Wu M, Madhani HD (2003) Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. Cell **112:** 725–736

Misson J, Raghothama KG, Jain A, Jouhet J, Block MA, Bligny R, Ortet P, Creff A, Somerville S, Rolland N, et al (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci USA **102:** 11934–11939

Mita S, Hirano H, Nakamura K (1997) Negative regulation in the expression of a sugar-inducible gene in *Arabidopsis thaliana*. A recessive mutation causing enhanced expression of a gene for beta-amylase. Plant Physiol **114:** 575–582

Niewiadomski P, Knappe S, Geimer S, Fischer K, Schulz B, Unte US, Rosso MG, Ache P, Flugge UI, Schneider A (2005) The *Arabidopsis* plastidic glucose 6-phosphate/phosphate translocator GPT1 is essential for pollen maturation and embryo sac development. Plant Cell **17:** 760–775

Nishizawa M, Komai T, Katou Y, Shirahige K, Ito T, Toh EA (2008) Nutrient-regulated antisense and intragenic RNAs modulate a signal transduction pathway in yeast. PLoS Biol **6:** 2817–2830

Oshima Y (1997) The phosphatase system in *Saccharomyces cerevisiae*. Genes Genet Syst **72:** 323–334

Persson BL, Lagerstedt JO, Pratt JR, Pattison-Granberg J, Lundh K, Shokrollahzadeh S, Lundh F (2003) Regulation of phosphate acquisition in *Saccharomyces cerevisiae*. Curr Genet **43:** 225–244

Raghothama KG (1999) Phosphate acquisition. Annu Rev Plant Physiol Plant Mol Biol **50:** 665–693

Raisner RM, Hartley PD, Meneghini MD, Bao MZ, Liu CL, Schreiber SL, Rando OJ, Madhani HD (2005) Histone variant H2A.Z marks the 5′ ends of both active and inactive genes in euchromatin. Cell **123:** 233–248

Richardson AE, Hadobas PA, Hayes JE (2001) Extracellular secretion of *Aspergillus phytase* from Arabidopsis roots enables plants to obtain phosphorus from phytate. Plant J **25:** 641–649

Rubio V, Linhares F, Solano R, Martin AC, Iglesias J, Leyva A, Paz-Ares J (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev **15:** 2122–2133

Sanchez-Calderon L, Lopez-Bucio J, Chacon-Lopez A, Cruz-Ramirez A, Nieto-Jacobo F, Dubrovsky JG, Herrera-Estrella L (2005) Phosphate starvation induces a determinate developmental program in the roots of *Arabidopsis thaliana*. Plant Cell Physiol **46:** 174–184

Santisteban MS, Kalashnikova T, Smith MM (2000) Histone H2A.Z regulates transcription and is partially redundant with nucleosome remodeling complexes. Cell **103:** 411–422

Shin H, Shin HS, Chen R, Harrison MJ (2006) Loss of At4 function impacts phosphate distribution between the roots and the shoots during phosphate starvation. Plant J **45:** 712–726

Sudarsanam P, Iyer VR, Brown PO, Winston F (2000) Whole-genome expression analysis of snf/swi mutants of *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA **97:** 3364–3369

Wippo CJ, Krstulovic BS, Ertel F, Musladin S, Blaschke D, Sturzl S, Yuan GC, Horz W, Korber P, Barbaric S (2009) Differential cofactor requirements for histone eviction from two nucleosomes at the yeast PHO84 promoter are determined by intrinsic nucleosome stability. Mol Cell Biol **29:** 2960–2981

Wykoff DD, Grossman AR, Weeks DP, Usuda H, Shimogawara K (1999) Psr1, a nuclear localized protein that regulates phosphorus metabolism in Chlamydomonas. Proc Natl Acad Sci USA **96:** 15336–15341

Yi K, Wu Z, Zhou J, Du L, Guo L, Wu Y, Wu P (2005) OsPTF1, a novel transcription factor involved in tolerance to phosphate starvation in rice. Plant Physiol **138:** 2087–2096

Zilberman D, Coleman-Derr D, Ballinger T, Henikoff S (2008) Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature **456:** 125–129